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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/09350 A3

(54) Title: GENETICALLY ENGINEERED BLEB VACCINE

(57) Abstract: The present invention relates to an immuno-protective and non-toxic Gram-negative bleb vaccine suitable for paediatric use. Examples of the Gram-negative strains from which the blebs are made are *N. meningitidis*, *M. catarrhalis* and *H. influenzae*. The blebs of the invention are improved by one or more genetic changes to the chromosome of the bacterium, including up-regulation of protective antigens, down-regulation of immunodominant non-protective antigens, and detoxification of the Lipid A moiety of LPS.

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/74 A61K39/02 C12N15/31 C12N15/67 A61K39/095
A61K39/116 A61K39/118 A61K39/09 A61K39/104 A61K39/102
A61K39/295 C07K14/195 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CLAASSEN I ET AL: "Production, characterization and control of a Neisseria meningitidis hexavalent class 1 outer membrane protein containing vesicle vaccine" VACCINE,GB,BUTTERWORTH SCIENTIFIC. GUILDFORD, vol. 14, no. 10, 1 July 1996 (1996-07-01), pages 1001-1008, XP004057632 ISSN: 0264-410X	1,3-5, 12,14, 15,35, 42,43, 54-56
Y	page 1001, column 1, line 1 -page 1002, column 1, last paragraph page 1004, column 1, paragraph 3 -column 2, paragraph 4 page 1006, column 2, paragraph 2 -page -/--	2,6-10, 20-23, 26-28, 36-40

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 March 2001	21.03.01
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Muller-Thomalla, K

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
	1007, paragraph 4 ---	
X	VAN DER LEY P ET AL: "Construction of Neisseria meningitidis strains carrying multiple chromosomal copies of the porA gene for use in the production of a multivalent outer membrane vesicle vaccine" VACCINE,BUTTERWORTH SCIENTIFIC. GUILDFORD,GB, vol. 13, no. 4, 1995, pages 401-407, XP004057740 ISSN: 0264-410X	1,3-5, 12,14, 15,35, 42,43, 54-56
Y	abstract page 401, column 1, line 1 -page 402, column 2, paragraph 4; figure 1 page 405, column 2, paragraph 2 -page 406, column 2, paragraph 2 ---	2,6-10, 13,16, 20-23, 26-28, 36-40
X	CARTWRIGHT K ET AL: "Immunogenicity and reactogenicity in UK infants of a novel meningococcal vesicle vaccine containing multiple class 1 (PorA) outer membrane proteins." VACCINE, (1999 JUN 4) 17 (20-21) 2612-9. , XP002154106	1,3-5, 12,14, 15,35, 42,43, 54-56
Y	abstract page 2613, column 1, last paragraph -column 2, last paragraph page 2617, column 2, paragraph 3 -page 2618, column 2, paragraph 1 ---	2,6-10, 13,16, 20-23, 26-28, 36-40
Y	HELMINEN M E ET AL: "A major outer membrane protein of moraxella catarrhalis is a target for antibodies that enhance pulmonary clearance of the pathogen in an animal model" INFECTION AND IMMUNITY,US,AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, vol. 61, no. 5, 1 May 1993 (1993-05-01), pages 2003-2010, XP002048787 ISSN: 0019-9567 abstract page 2008, column 2, paragraph 2 -page 2009, column 2, paragraph 2 ---	2,6-10, 13,16, 20-23, 26-28, 36-40

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Inte. ional Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 098 997 A (ANILIONIS ALGIS ET AL) 24 March 1992 (1992-03-24) abstract column 3, line 31 -column 6, line 35 column 7, line 11 -column 9, line 40 column 18, line 19 -column 29, line 63 column 23, line 51 -column 25, line 55 column 32, line 8 -column 33, line 16	2,6-10, 13,16, 20-23, 26-28, 36-40
Y	US 4 666 836 A (INOUYE MASAYORI ET AL) 19 May 1987 (1987-05-19) column 6, line 11-51 column 15, line 14 -column 16, line 5; claims 2,44-46	1-10, 13-15, 20-22, 26,27, 35-40, 42,54-56
Y	US 5 888 722 A (CHEN MING ET AL) 30 March 1999 (1999-03-30) column 5, line 1 -column 6, line 35; claims 1-20	1-10, 13-15, 20-22, 26,27, 35-40, 42,54-56
X	VAN LOON F P L ET AL: "Field trial of inactivated oral cholera vaccines in Bangladesh: results from 5 years of follow-up" VACCINE,GB,BUTTERWORTH SCIENTIFIC. GUILDFORD, vol. 14, no. 2, 1 February 1996 (1996-02-01), pages 162-166, XP004057360 ISSN: 0264-410X abstract page 162, column 1, paragraph 1 page 164, column 2, last paragraph	43

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Int. ional Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	KYD J M ET AL: "Killed whole bacterial cells, a mucosal delivery system for the induction of immunity in the respiratory tract and middle ear: an overview" VACCINE,GB,BUTTERWORTH SCIENTIFIC. GUILDFORD, vol. 17, no. 13-14, January 1999 (1999-01), pages 1775-1781, XP004158321 ISSN: 0264-410X abstract page 1779, column 1, line 1 -page 1780, column 1, paragraph 1 ---	43
X	LUBITZ W ET AL: "Extended recombinant bacterial ghost system" JOURNAL OF BIOTECHNOLOGY,NL,ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, vol. 73, no. 2-3, 20 August 1999 (1999-08-20), pages 261-273, XP004180188 ISSN: 0168-1656 abstract page 262, column 1, last paragraph page 267, column 1, paragraph 2 -page 268, column 1, paragraph 1 page 270, column 1, paragraph 2 -column 2, paragraph 1 ---	43
A	WO 99 10497 A (LEY PETER ANDRE V D ;NEDERLANDEN STAAT (NL); STEEGHS LIANA JULIANA) 4 March 1999 (1999-03-04) the whole document ---	
A	WO 98 33923 A (MASKELL DUNCAN JOHN ;DOUGAN GORDON (GB); IMPERIAL COLLEGE (GB)) 6 August 1998 (1998-08-06) the whole document -----	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 00/07424

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 55 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [X] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

Part of claims 1-10,12-15,20-22,26,27,35-40,42,54-56 and whole of claims 16,23,28 and 43

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: Part of claims 1-7,12-15,20-22,26,27,35-40,42, 54-56 and whole of claims 16,23,28

Invention 1
A genetically-engineered "bleb" preparation from a Gram-negative bacterial strain obtainable by a process of reducing immunodominant variable or non-protective antigens within the bleb preparation comprising the steps of determining the identity of such antigen, engineering a bacterial strain to produce less or none of said antigen, and making blebs from said strain. A modified Gram-negative bacterial strain from which the bleb preparation is made.

2. Claims: Part of claims 1-10,13-15,20-22,26,27,35-40,42, 54-56

Invention 2
A genetically-engineered "bleb" preparation from a Gram-negative bacterial strain obtainable by a process of upregulating expression of protective OMP antigens within the bleb preparation comprising the steps of identifying such antigen, engineering a bacterial strain so as to introduce a stronger promoter sequence upstream of a gene encoding said antigen such that said gene is expressed at a level higher than in the non-modified bleb, and making blebs from said strain. A modified Gram-negative bacterial strain from which the bleb preparation is made.

3. Claims: Part of claims 1-7,13-15,20-22,26,27,35-40,42, 54-56 and whole of claims 11,19

Invention 3
A genetically-engineered "bleb" preparation from a Gram-negative bacterial strain obtainable by a process of upregulating expression of conditionally-expressed, protective OMP antigens within the bleb preparation comprising the steps of identifying such an antigen, engineering a bacterial strain so as to remove the repressive control mechanisms of its expression, and making blebs from said strain. A modified Gram-negative bacterial strain from which the bleb preparation is made.

4. Claims: Part of claims 1-7,12-15,20-22,26,27,35-40,42, 54-56 and whole of claims 17,24,29

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Invention 4
A genetically-engineered "bleb" preparation from a Gram-negative bacterial strain obtainable by a process of modifying lipid A portion of bacterial LPS within the bleb preparation, comprising the steps of identifying a gene involved in rendering the lipid A portion of LPS toxic, engineering a bacterial strain so as to reduce or switch off expression of said gene, and making blebs from said strain. A modified Gram-negative bacterial strain from which the bleb preparation is made.

5. Claims: Part of claims 1-10,13-15,20-22,26,27,35-40,42, 54-56 and whole of claims 18,25,30

Invention 5
A genetically-engineered "bleb" preparation from a Gram-negative bacterial strain obtainable by a process of modifying lipid A portion of bacterial LPS within the bleb preparation, comprising the steps of identifying a gene involved in rendering the lipid A portion of LPS less toxic, engineering a bacterial strain so as to introduce a stronger promoter sequence upstream of said gene such that said gene is expressed at a level higher than in the non-modified bleb, and making blebs from said strain. A modified Gram-negative bacterial strain from which the bleb preparation is made.

6. Claims: Part of claims 1-7,13-15,20-22,26,27,35-40,42, 54-56 and whole of claims 31-34

Invention 6
A genetically-engineered "bleb" preparation from a Gram-negative bacterial strain obtainable by a process of reducing lipid A toxicity within the bleb preparation and increasing the levels of protective antigens, comprising the steps of engineering the chromosome of a bacterial strain to incorporate a gene encoding a Polymyxin A peptide, or a derivative or analogue thereof, fused to a protective antigen, and making blebs from said strain. A modified Gram-negative bacterial strain from which the bleb preparation is made.

7. Claims: Part of claims 1-7,13-15,20-22,26,27,35-40,42, 54-56

Invention 7
A genetically-engineered "bleb" preparation from a Gram-negative bacterial strain obtainable by a process of creating conserved OMP antigens on the bleb preparation

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

comprising the steps of identifying such antigen, engineering a bacterial strain so as so delete variable regions of a gene endoding said antigen, and making blebs from said strain. A modified Gram-negative bacterial strain from which the bleb preparation is made.

8. Claim : Part of claim 54

Invention 8
A genetically-engineered "bleb" preparation from a Gram-negative bacterial strain obtainable by a process of reducing expression within the bleb preparation of an antigen which shares a structural similarity with a human structure and may be capable of inducing an auto-immune response in humans, comprising the steps of identifying a gene involved in the biosynthesis of the antigen, engineering a bacterial strain so as to reduce or switch of expression of said gene, making blebs from said strain.

9. Claim : part of claim 54

Invention 9
A genetically-engineered "bleb" preparation from a Gram-negative bacterial strain obtainable by a process of upregulating expression of protective OMP antigens within the bleb preparation comprising the steps of identifying such antigen, engineering a bacterial strain so as to introduce into the chromosome one or more further copies of a gene encoding said antigen controlled by a heterologous, stronger promoter sequence, and making blebs from said strain..

10. Claim : 43

Invention 10
An immuno-protective and non-toxic Gram-negative bleb, ghost or killed whole cell vaccine suitable for paediatric use.

11. Claims: Part of claims 41,44-47

Inventions 11-88
Isolated polynucleotide sequence which hybridizes under highly stringent conditions to at least a 30 nucleotide portion of the nucleotide sequence No. 2 or a complementary strand thereof and its use in performing a homologuous recombination event within 1000 bp upstream of Gram-negative bacterial chromosomal gene in order to either increase or decrease expression of said gene.

..ibidem for sequences No. 3-23,25 and 27-81, which correspond to inventions 12-88 and vectors containing the

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

same.

12. Claims: Claims 48-53

Invention 89
A process of genetically upregulating expression of a gene withing a Gram-negative bacterial strain comprising the steps making a vector comprising a strong promoter sequence and a nucleotide sequence which is capable of recombination with a sequence of at least 30 nucleotides in the 1000bp upstream of the (undefined) gene, transforming a bacterial strain with the vector, and performing a homologuous recombination event between the chromosome and the vector to introduce the promoter within 1000bp upstream of the initiation codon of the (undefined) gene. Modified bacterial strain obtainable by this method.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. ional Application No
PCT/EP 00/07424

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